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Channeling of Ammonia in Glucosamine-6-phosphate Synthase

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Glucosamine-6-phosphate synthase catalyses the first and rate-limiting step in hexosamine metabolism, converting fructose 6-phosphate into glucosamine 6-phosphate in the presence of glutamine. The crystal structure of the *Escherichia coli* enzyme reveals the domain organisation of the homodimeric molecule. The 18 Å hydrophobic channel sequestered from the solvent connects the glutaminase and isomerase active sites, and provides a means of ammonia transfer from glutamine to sugar phosphate. The C-terminal decapeptide sandwiched between the two domains plays a central role in the transfer. Based on the structure, a mechanism of enzyme action and self-regulation is proposed. It involves large domain movements triggered by substrate binding that lead to the formation of the channel.

Keywords: glutamine amidotransferase; glucosamine-6P synthase; crystal structure; ammonia channel

Introduction

Glutamine-dependent amidotransferases are responsible for utilisation of the amide nitrogen of glutamine in a variety of biosynthetic reactions.[1–3] Given a central role played by these enzymes in cellular metabolism, elucidation of their structure and mechanism of action may have significant implications for the discovery of new therapeutic agents. Glucosamine-6-phosphate synthase (GlmS, L-glutamine:D-fructose-6P amidotransferase, EC 2.6.1.16) catalyses the first step in hexosamine metabolism, converting fructose 6-phosphate (Fru6P) into glucosamine 6-phosphate (GlcN6P) in the presence of glutamine (Figure 1). The reaction catalysed by GlmS is irreversible, and is therefore considered as a committed step. The end product of the pathway, *N*-acetylglucosamine, is an essential building block of bacterial and fungal cell walls. Structural differences between prokaryotic and human enzymes may be exploited to design specific inhibitors, which may serve as prototypes of anti-fungal and anti-bacterial drugs.[4] In mammalian cells, GlmS is an insulin-regulated enzyme, which controls the flux of glucose into the hexosamine pathway.[5] As the rate-limiting enzyme of mucus synthesis, GlmS attracts much attention as a potential drug target for treatment of gastric disorders.[6]

The GlmS monomer is composed of two structurally and functionally distinct domains.[7] The N-terminal 30 kDa glutaminase domain catalyses hydrolysis of glutamine to glutamate and ammonia. The C-terminal 40 kDa isomerase domain utilises this ammonia for the conversion of Fru6P to GlcN6P. The reaction is thought to proceed through the formation of fructosimine 6-phosphate (FruN6P), which is then isomerised to GlcN6P.[8] Unlike most other amidotransferases, GlmS cannot use exogenous ammonia as a nitrogen donor.[7] The two domains separated by controlled chymotrypsin proteolysis, or expressed separately, retain their ability to bind substrates and to catalyse, respectively, glutamine hydrolysis and sugar isomerisation.[9,10] The three-dimensional structures of both domains of *Escherichia coli* GlmS have been

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Abbreviations used: GlmS, glucosamine-6-phosphate synthase; Fru6P, fructose 6-phosphate; FruN6P, fructosimine 6-phosphate; Glc6P, glucose 6-phosphate; GlcN6P, glucosamine 6-phosphate; GlcNol6P, 2-amino-2-deoxy-glucitol 6-phosphate; DON, 6-diazo-5-oxo-L-norleucine; PurF, phosphoribosyl pyrophosphate amidotransferase; CPS, carbamoyl phosphate synthetase; AsnB, asparagine synthetase; GltS, glutamate synthase; Ntn, N-terminal nucleophile.

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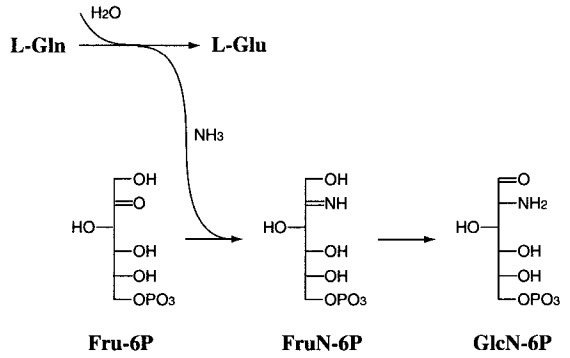

Figure 1. The reaction catalysed by GlmS. Ammonia produced by glutamine hydrolysis is utilised for sugar phosphate amination.

determined by X-ray crystallography:[11,12] the glutaminase domain in complex with glutamate (PDB code 1GDO) and with γ-glutamyl hydroxamate (code 1GMS), the isomerase domain in complex with GlcN6P (code 1MOQ), glucose 6-phosphate (Glc6P) (code 1MOR), and 2-amino-2-deoxy-glucitol 6-phosphate (GlcNol6P) (code 1MOS). These structures revealed the mechanism of glutamine hydrolysis as that of the N-terminal nucleophile (Ntn) type, and suggested a mechanism of glutamine-dependent Fru6P isomerisation through an enol/enediol intermediate similar to that of triosephosphate isomerase.[13]

Now we have solved the crystal structure of the intact protein, which is essential for understanding of how the ammonia produced at the glutamine site is delivered to the sugar phosphate site. Crystallographic studies of several amidotransferases[14–19] indicated formation of a solvent-shielded ammonia channel as a means of nitrogen transfer. The structure of GlmS presented here reveals a similar channel between the glutamine and the sugar phosphate sites, which are separated by 18 Å. The structure shows the oligomeric organisation of the protein molecule and, in conjunction with the previously determined crystal complexes, allows us to propose a mechanism of GlmS action.

Results and Discussion

Quaternary structure

The polypeptide chain of GlmS is folded into two distinct domains (Figure 2). Residues 1 to 239 comprise the glutaminase domain responsible for the hydrolysis of glutamine. The domain has a four-layer structure with two antiparallel β-sheets sandwiched between layers of α-helices. This fold is characteristic of the superfamily of Ntn hydrolases,[20] which includes glutamine amidotransferases, penicillin acylase, aspartyl glucosaminidase, and 20S proteasome. The N-terminal catalytic cysteine residue in GlmS is located at the beginning of the central β-strand in a shallow cleft on the surface of the molecule. Other amino acid residues involved in substrate binding and catalysis reside in the loops connecting β-strands. One of the loops (residues 73-80, the Q-loop) is of particular importance, as it covers the active site upon binding of glutamine, and thus shields it from the solvent.

The isomerase domain of GlmS comprises residues 249-608. It is responsible for the binding of Fru6P and its conversion to GlcN6P. The domain is

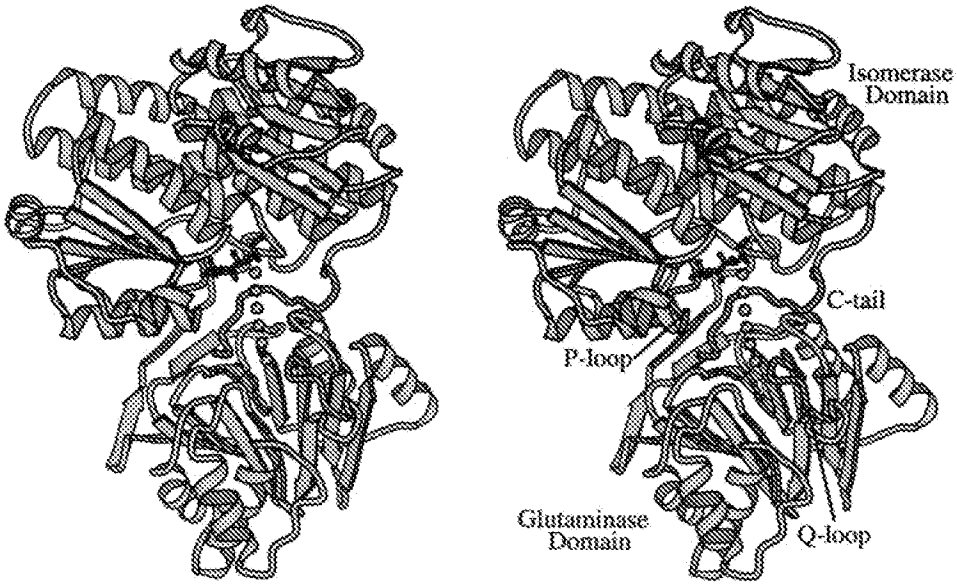

Figure 2. Stereo view of the GlmS monomer. The functionally important P-loop, Q-loop, and C-tail are shown in magenta. Glc6P is shown as a ball-and-stick model. The ammonia channel is indicated by cyan spheres.

bilobal in shape and consists of two topologically identical subdomains of equal size, each of which has a canonical nucleotide-binding fold characterised by a five-stranded parallel β-sheet flanked by α-helices on both sides. The strand order is 3-2-1-4-5, with all connections between strands being right-handed. The active site is located in the N-terminal half of the domain at the carboxyl edge of the β-sheet. Amino acid residues implicated in catalysis are distributed over a number of secondary structural elements, which belong to both halves of the domain. One residue, His504*, which is believed to play a key role in the sugar ring opening, is on a different polypeptide chain (therefore marked with an asterisk). Hence, for the active site to be assembled, dimerisation of the protein is required (Figure 3).

A ten residue linker between domains has a well-defined conformation in the crystal structure, although it does not form any secondary structure, and is not closely associated with either of the domains. This feature is indicative of possible relative movements of the domains. A flexible linker would provide the necessary freedom for such movements.

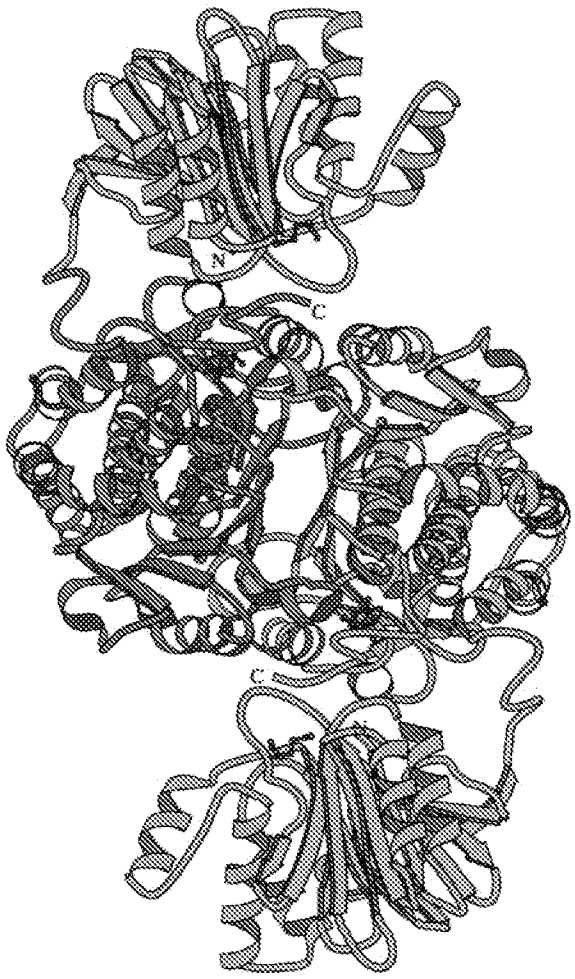

Figure 3. GlmS dimer as viewed along the molecular 2-fold axis. Glutaminase domains are shown in green, isomerase domains are red and yellow. Active sites are indicated by ball-and-stick models of the substrates.

The structure of the intact GlmS presented here displays the arrangement of functional domains in a homodimeric molecule (Figure 3). The core of the molecule is a dimer of the isomerase domains. It has a spherical shape with a diameter of about 60 Å. This dimer is the same as was observed in the crystal structure of the isomerase function of GlmS in spite of a completely different crystallographic environment. Moreover, there are two crystallographically independent GlmS dimers in the present structure, and both have the same quaternary structure. Obviously, this dimer represents the functional unit of the enzyme. The molecular symmetry is very close to the exact dyad as can be seen from comparison of the "symmetric" and "asymmetric" dimers. The r.m.s. deviation of the isomerase dimer from the exact symmetry is 0.7 Å in the present structure (calculated over all 360 C^{α} atoms).

Glutaminase domains are on the opposite sides of the core dimer. This makes the molecule look like an ellipsoid with the axes ratio of 2:1. The overall size of the GlmS molecule is 115 Å × 65 Å × 50 Å. The crystal structure resolves a question about the spatial arrangement of the glutaminase and isomerase domains within a GlmS dimer. Theoretically, there are two possibilities. One assumes that ammonia, the reaction intermediate, is transferred between domains of the same polypeptide, while the other assumes that the transfer occurs between different subunits. The present structure is in favour of the first possibility. In other words, both substrates, Gln and Fru6P, involved in the reaction, bind to the same protein monomer.

Dimerisation buries 3000 $Å^2$ of the protein surface, 90% of which is between the isomerase domains. The interface between the isomerase and the glutaminase domains of the same monomer involves the C-terminal decapeptide (referred to as the C-tail) and residues around Cys1. In particular, the C-tail provides two pockets for aromatic groups of Tyr28 and Trp74 of the glutaminase domain (Figure 4). Tyr28 fits the hydrophobic pocket formed by residues 597-602, while its hydroxyl group binds the backbone carbonyl of Pro598. There is also a hydrophobic cluster at the domain interface, which includes Tyr25, Leu194, Leu238, Tyr251, Thr395, and Ile397. The only cross-contacts between the glutaminase and the isomerase domains of different monomers are the H bonds between Arg539* and two groups at the glutamine site, namely the carbonyl group of Ala75 and the carboxyl group of Asp29. The arrangement of domains in the GlmS molecule indicates that the conformational flexibility, if required, may be achieved at the expense of the minimal isomerase/

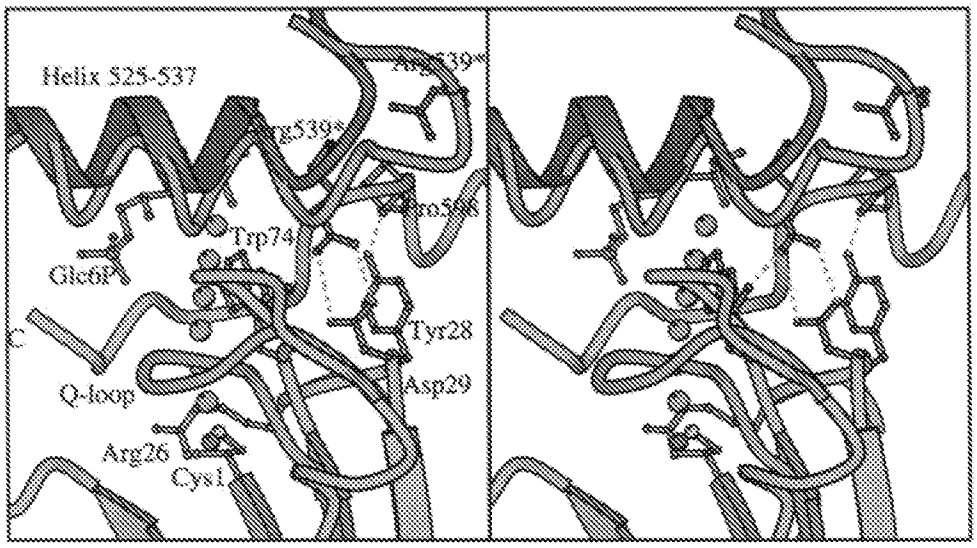

Figure 4. Stereo view of the conformational rearrangements in GlmS upon substrate binding. Domains of the present structure are coloured as in Figure 3. Superimposed (in cyan) are the Q-loop of the inhibited glutaminase domain (code 1GDO) and helix 526-539 of the isolated isomerase domain (code 1MOQ). Arg539* is shown for both conformations of the helix. The ammonia channel is indicated by cyan spheres, the amido nitrogen atom of Gln by a small blue sphere. H bonds are indicated by broken lines.

glutaminase interface rather than by disruption of the isomerase dimer.

Isomerase domain

The enzyme was crystallised in the presence of one of the substrates, Fru6P. The electron density indicates a ligand molecule bound at the sugar phosphate site (Figure 5). The phosphate group is embraced by the loop of residues 347-352 (P-loop) in a manner observed in crystal complexes of the isomerase domain. The phosphate oxygen atoms form H bonds to the hydroxyl groups of Ser347, Ser349, Thr352, and to the main-chain amino groups of Ser349 and Gln348. The sugar moiety is unexpectedly found in an open conformation. The hydroxyl groups of the sugar form H bonds to the protein: O-1 to the carboxyl group of Glu488 and

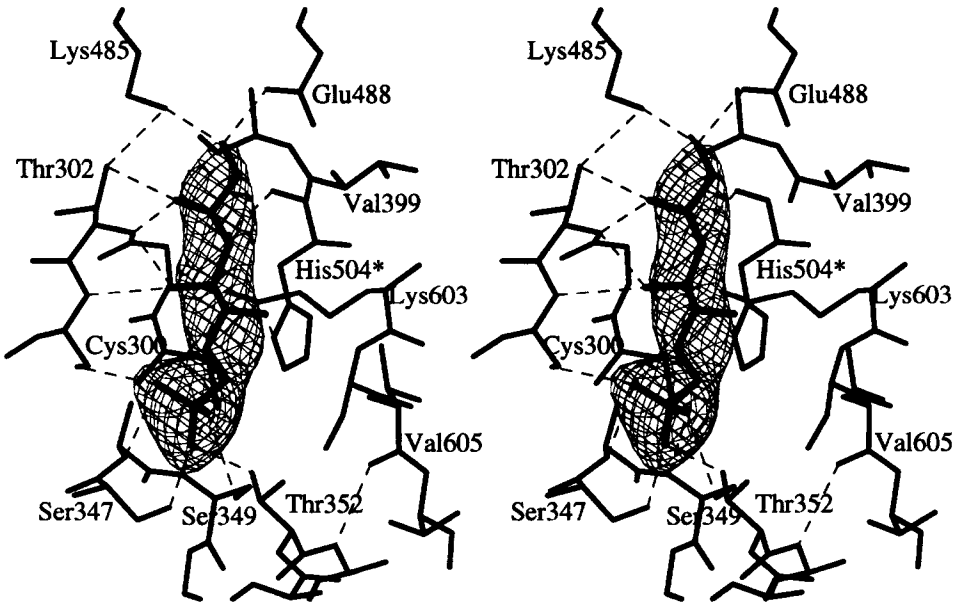

Figure 5. Stereo view of the sugar phosphate site. Glc6P and surrounding residues are shown by continuous lines, H bonds by broken lines. The $(2F_o - F_c)$ electron density at Glc6P is contoured at the 2σ level.

to the amino group of Lys485; O-2 and O-4 to Thr302; O-3 and O-5 to the carbonyl and imidazole amino groups of His504*, respectively.

A similar pattern of hydrogen bonds has been observed for the transition state analogue GlcNol6P bound to the isomerase domain. This pattern is in contrast to the binding of the closed form of the sugar, which was observed in complexes with Glc6P and GlcN6P. Cyclic sugars do not lie so deep in the binding pocket and do not form direct H bonds to the catalytic Glu488, whereas open-chain sugars interact with Glu488 but not with the C-tail.

Because of the moderate resolution of the X-ray data, the present structure provides no experimental evidence of whether Fru6P has been converted into Glc6P. In our previous studies, crystallisation of the isolated isomerase domain with Fru6P resulted in the structure where the substrate was found to be isomerised and in a cyclic form (Glc6P). Given that the substrate was processed to the open chain form in the present experiment, there are no visible obstacles for the isomerisation to take place, as all the catalytic elements are there and ready to act. The cyclisation of the product, however, requires considerable room, which may be not available in the closed active site. It seems possible that the product was trapped right after the isomerisation step when further movements of the domains were constrained by the crystal lattice.

The overall structure of the isomerase domain in the intact protein is very similar to that determined for the isolated domain. Their superposition reveals a major difference in the position of helix 526-539 (Figure 4), whereas the rest of the structure remains the same. The shift of the helix is observed in all three independent monomers in the present structure. The helix is not involved in crystal contacts and therefore the differences are likely caused by the interaction with the glutaminase domain. The r.m.s. deviation of all 360 C^{α} atoms is 1.6 Å when compared to the isolated domain (code 1MOQ). However, it drops to 0.7 Å when the helix is excluded from the calculation. The helix is shifted by one turn, i.e. by 6 Å, towards its C-end in the isolated domain. In the absence of the glutaminase domain, the guanidinium group of Arg539* makes H bonds to the backbone carbonyl group of Ile510* and to the amido group of Asn600 of another isomerase domain, whereas in the present structure it binds the carbonyl group of Ala75 and the carboxyl group of Asp29 of the glutaminase domain. The two binding sites of Arg539* are separated by 8 Å. Interaction of Arg539* with the glutaminase domain is considered as an important element of the enzymatic mechanism.

Glutaminase domain

The glutamine site in the present structure is empty because no substrate was added to the crystallisation solution. The Q-loop, which covers the active site in complexes with Glu and Glu-hydroxamate, is in an open conformation, so that the binding pocket is exposed to solvent. Comparison of the structures of the glutaminase domain in different states shows that the conformational changes accompanying substrate binding are limited to two neighbour loops, the Q-loop and the loop of residues 25-29 (Figure 4). No significant difference is detected for the rest of the domain. The r.m.s. deviation calculated for all 238 C^{α} atoms is 1.1 Å (*versus* code 1GDO). A similar rearrangement at the active site was observed in phosphoribosyl pyrophosphate amidotransferase (PurF) upon binding of a glutamine analogue.[16]

Superposition of the apo-protein and the inhibited glutaminase domain indicates that the Q-loop moves by about 6 Å as measured for C^{α} of His77 on top of the loop, and covers the active site. Arg73 anchors the carboxylate group of the substrate, while the hydroxyl group of Thr76 binds the amino group. The carbonyl group of Trp74 helps to orient the amido group of Gln for a nucleophilic attack by Cys1. In an "open" conformation, the Q-loop is H-bonded to Arg539*.

A small but significant difference between the two structures is the rotation of the catalytic Cys1 around the C^{α}-C bond. In the inhibited glutaminase domain, Cys1 is fixed in an inactive conformation, with the terminal amino group pointing towards the ligand and SH-group away from the binding site. In the present structure, Cys1 is in the active conformation, which is probably supported by the interaction with the isomerase domain. Arg26 behind the catalytic Cys1 may play a key role in the communication with the other domain. Arg26 binds the N-terminal amino group by the backbone carbonyl group, and thus prevents the rotation of Cys1. In addition, the side-chain of Arg26 is quite close to Cys1 leaving no room for the sulfhydryl group. The conformation of Arg26, is in turn, defined by its interaction with the C-tail. As a result, Cys1 is fixed in the active conformation. This mechanism may explain a dramatic (130-fold in k_{cat}) activation of glutamine hydrolysis in the presence of Fru6P.[8] Binding of Fru6P causes a rearrangement of the C-tail that is relayed to Cys1 *via* Arg26.

Intramolecular channel

Ammonia produced by glutamine hydrolysis should remain trapped in the enzyme to avoid protonation and to retain its nucleophilic character. The crystal structure of GlmS presents an internal channel between the two active sites, which are separated by 18 Å. The walls of the channel are aligned by hydrophobic side-chains that allow diffusion of NH_3 between the two sites. Both isomerase and glutaminase domains contribute to the formation of the channel. The central portion of the channel resides in the C-terminal loop 601-607 (Figure 6). Leu601, Ala602, Val605, and Val607 of this loop form a hydrophobic entrance to the sugar-binding site. The glutaminase "contribution"

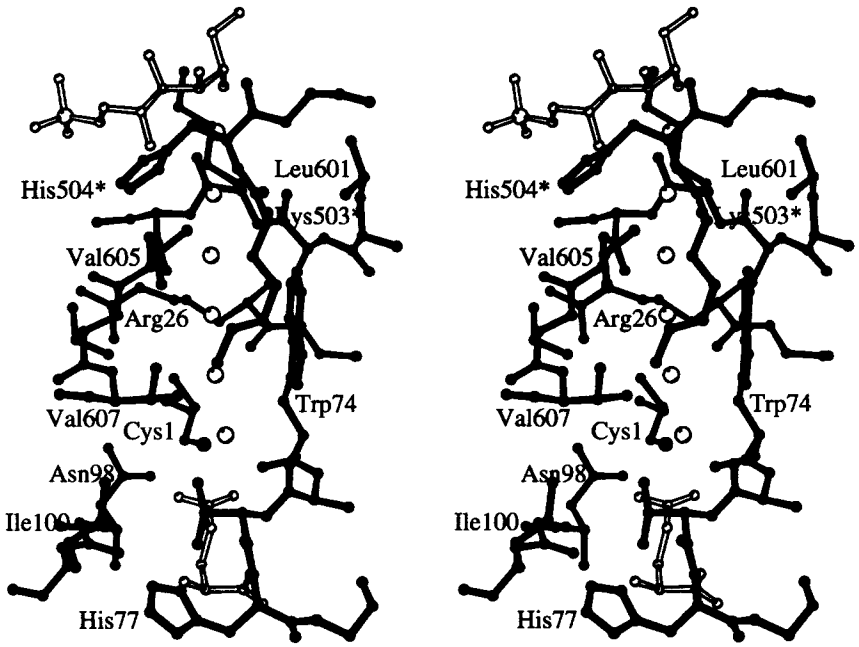

Figure 6. Stereo view of the ammonia channel in GlmS. The channel is indicated by white spheres. Substrates are shown in open lines, Glc6P is as observed in the present structure, Gln as observed in the structure of the glutaminase domain.

to the channel is the side-chains of Trp74 and Arg26. A third party involved is the histidine loop (H-loop) of another protein subunit that bears the catalytic His504*. The tripeptide 503*-505* and the aliphatic chain of Lys503* form one wall of the channel. All these residues are strictly conserved in the GlmS sequence.

In the present structure, the channel remains accessible to the solvent at the glutamine site. There is no substrate bound there, and the Q-loop is in the open conformation. Superposition of the glutaminase domain in a closed conformation (code 1GDO) on the present structure shows that the loop would isolate the channel from solvent completely (Figure 6). The diameter of the channel is about 6 Å as measured between atom centres on the opposite sides of the channel. However, the path is blocked by the indole ring of Trp74, leaving almost no clearance. A possible domain rearrangement leading to the formation of a fully open ammonia channel is discussed in the next section.

An intramolecular channel connecting the glutaminase site with the synthetic site seems to be a common feature of glutamine-dependent amidotransferases. Such channels have been observed in, or predicted from the crystal structures of carbamoyl phosphate synthetase (CPS),[14] GMP synthetase,[15] PurF,[16] anthranilate synthase,[17] asparagine synthetase (AsnB),[18] and glutamate synthase (GltS).[19] The channels are formed predominantly by hydrophobic residues and polypeptide backbone atoms. Their length varies from 15 to 45 Å (the longest is in CPS). While the synthetic domains in these enzymes are quite different, the domains responsible for the hydrolysis of glutamine belong to one of the two types. GlmS has the glutaminase domain of the Ntn type characterised by the N-terminal catalytic cysteine residue. This group includes PurF, AnsB and GltS. The diversity of synthetic domains in amidotransferases implies that ammonia channels are formed by different structural elements. In GlmS, the central element is the C-terminal decapeptide (C-tail), which may serve as a sequence fingerprint of the enzyme. The amino acid sequence of this fragment is conserved in all organisms from archaea to mammals. Like GlmS, significant conformational changes accompanying substrate binding are required for the formation of a channel in most amidotransferases. This is in contrast to CPS, where the channel exists in the apo protein.

The amidotransferase family is an example of both the divergent and convergent evolution of proteins. On the one hand, the glutaminase units have diverged from one of two common origins, the Ntn and the "triad" type. On the other hand, pairwise combinations of the glutaminase units with various synthetic units have all converged to the enzymes with the intramolecular channel as a means of nitrogen transfer.

Mechanism of nitrogen transfer

The structure of the intact protein reported here, as well as the structures of the enzyme-product complexes of both domains determined earlier, allow us to propose a mechanism of enzyme action and explain available kinetic data. Detailed catalytic mechanisms of glutamine hydrolysis and sugar phosphate isomerisation by the corresponding domains have been described by Isupov *et al.*[11] and by Teplyakov *et al.*,[13] respectively. They are

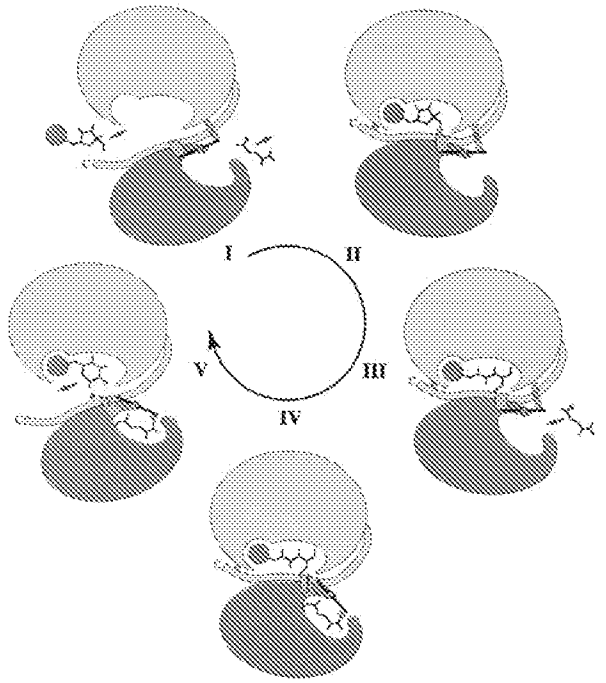

Figure 7. The catalytic cycle of GlmS. Only one protein monomer is shown (isomerase domain yellow, glutaminase domain green). The phosphate group of Fru6P and GlcN6P is shown as an orange sphere, the amino group as a small blue sphere, the water molecule as a red sphere. Essential H bonds are shown as broken red lines. The swinging gate at the glutamine site represents the Q-loop. Step I: both active sites are open, the Q-loop is anchored by Arg539*, Trp74 blocks the ammonia channel. Step II: the C-tail closes the Fru6P site. Step III: following the sugar ring opening, a Schiff base with Lys603 is formed. Step IV: Gln enters, the Q-loop closes the site, Trp74 opens the channel, ammonia enters the sugar site. Step V: sugar phosphate is aminated and isomerised, the site opens upon cyclisation of GlcN6P, water molecule enters the channel, deacylation of a Glu thioester takes place.

incorporated in a general mechanism proposed here (Figure 7).

At first, let us consider how the protein can bind the sugar phosphate substrate. Crystal structures of the isomerase domain with various ligands show the "closed" conformation of the active site. For the site to open, the C-tail should move away. This would provide an opening of at least 10 Å as measured between Thr352 and Val399 located on the opposite sides of the binding pocket (Figure 5). Removal of the H-loop with His504* would not open the entrance wide enough for a bulky substrate. Besides, this second possibility seems unlikely, as it would require a disruption of a significant part of the dimer interface. Dissociation of the isomerase domains is energetically costly as compared to the displacement of the C-tail. Whereas the extensive interface between isomerase domains covers 20 % of their surface, there is only one direct H bond that links the C-tail to the isomerase domain; namely, that between the 605 carbonyl group and the 353 amino group of the P-loop. One can say that the C-tail is positioned at the sugar site by the interaction with the substrate rather than with the protein.

To open the active site, the C-terminal decapeptide needs to rotate around the C^{α}-C bond of Pro598 by just 20 °. This movement will not disrupt contacts of the polypeptide fragment upstream Pro598. Moreover, it seems that proline is conserved in this position specifically for this purpose. The constrained main-chain torsion angle ϕ of a proline provides the necessary rigidity and makes it an ideal hinge point.

Such a rigid-body movement of the C-tail implies that the interface with the glutaminase domain may be preserved even in the absence of Fru6P. The key anchors are the side-chains of Tyr28, Arg26, and Trp74. We further speculate that at this step (Figure 7, step I), when the sugar phosphate site is open, the only interaction between domains, is probably the link between the Q-loop and Arg539*, which helps to keep the glutaminase site open until glutamine is bound. This may explain the much lower K_m(Gln) for GlmS as compared to that of the isolated domain,[9] where the Q-loop may occasionally close the binding site.

Binding of Fru6P causes the closure of the isomerase site by the C-tail (Figure 7, step II). The 602 and 603 carbonyl groups form direct H bonds to the sugar hydroxyl groups, while the 605 amino group interacts through a water molecule. The glutamine site remains open. The intramolecular channel is now formed, although Trp74 blocks it. An ordered binding of substrates with the sugar phosphate group binding prior to the amino acid substrate was deduced from the product and dead-end inhibition studies[21] using 6-diazo-5-oxo-L-norleucine (DON), a competitive inhibitor of glutaminase.

Fru6P bound to the protein undergoes the ring opening catalysed by His504*. As a result of the conformational change in the sugar, H bonds to the C-tail are lost, and instead a Schiff base with Lys603 is formed[22] (Figure 7, step III). In the "synthetic" pathway of the reaction, the incoming ammonia ion replaces Lys603 in the Schiff base. FruN6P undergoes isomerisation to GlcN6P catalysed by Glu488. In the absence of ammonia, a small fraction of the substrate may proceed directly to the isomerisation step without forming a Schiff base. This unfavourable activity of GlmS is 10^5 times lower than the synthase activity.[10] In accordance with the proposed mechanism, mutation of Lys603 to Arg results in a decrease of the synthase activity and increase of the isomerase activity.[8]

Binding of glutamine triggers another cascade of conformational changes in the protein (Figure 7, step IV). The Q-loop closes the active site by forming numerous H bonds to the substrate. Driven by

this rearrangement, the indole ring of Trp74, which is part of the Q-loop, is displaced from the channel. The shift of Trp74 is indicated by the superposition of the Q-loop in a closed conformation (as in the complex with Glu) on the present structure. Ammonia produced by the hydrolysis of glutamine is transferred to the sugar phosphate site, and the sugar is aminated at C-2.

Deacylation of a glutamyl thioester adduct requires a water molecule. However, the glutamine site is completely shielded from the solvent. The sugar site is likely to open first to allow the cyclisation of GlcN6P. Dissociation of the C-tail from the isomerase domain opens the ammonia channel to the solvent. A water molecule required for deacylation can enter the glutaminase site through this channel (Figure 7, step V). The release of glutamate from the active site follows the displacement of the Q-loop and is probably driven by the repulsion between the carboxyl group of Glu and the sulfhydryl group of Cys1.

Crystal structures of GlmS complexes with various ligands reflect different conformational states of the protein. The structure presented here corresponds to the intermediate step with Fru6P bound and isomerised to Glc-6P, the Gln site being vacant and open, and the ammonia channel being formed but blocked by Trp74. Structures of the isolated domains with the reaction products show the closed conformations of the active sites as they are expected to be in the enzyme-substrate complexes. The three-dimensional information is still missing several key steps, such as the substrate-free state and the ternary complex with both substrates. More experimental data are needed to verify our hypothesis.

The proposed mechanism provides an explanation to the puzzling fact that GlmS cannot use exogenous ammonia for GlcN6P synthesis.[7] The answer is in the structure of the intramolecular channel. Fru6P bound to the protein can be approached by ammonia only through the channel, which is blocked by Trp74. This obstacle is removed upon binding of glutamine, but then the glutamine site is closed and the channel is shielded from the solvent. Remarkably, a similar gating mechanism was observed in tryptophan synthase.[23] Phe280 partially blocks the intramolecular channel in the presence of Na^+, but swings out of the channel when Na^+ is replaced with K^+.

Materials and Methods

Crystallisation and data collection

GlmS was overexpressed in *E. coli*, purified to homogeneity and crystallised as described.[24] The crystals grew from 10 mg/ml protein solution in 0.1 M Hepes (pH 7.0), 1 M LiCl, 3% (w/v) PEG 4000, 10 mM Fru6P. Well-shaped isometric crystals reached their maximum size of 0.3 mm × 0.3 mm × 0.3 mm in few weeks. They belong to the monoclinic space group C2 with $a = 131.4$ Å, $b = 112.4$ Å, $c = 185.1$ Å, $\beta = 96.4°$.

The crystals were soaked in a cryo-solution consisting of the well solution plus 30% (v/v) glycerol, and flash-frozen in a stream of nitrogen at 100 K. X-ray diffraction data to 3.1 Å were collected at the EMBL synchrotron beamline X11 (DESY, Hamburg) using a Mar imaging plate detector, and processed with DENZO and SCALEPACK.[25] Data statistics are given in Table 1.

Structure determination and refinement

The structure was solved by molecular replacement with the program MOLREP[26] using high-resolution atomic models of both GlmS domains (codes 1GDO and 1MOQ). The initial search model consisted of a dimer of the isomerase domains as it was observed in the complex with GlcN6P (code 1MOQ). This dimer was believed to represent the functional core of the intact protein.[12] Rotational and translational searches have produced one dimer in a general position (referred to as asymmetric) and another dimer (symmetric) on a crystallographic 2-fold axis. The orientation of the molecular axis of the asymmetric dimer was consistent with the self-rotation function, which featured one major peak at $\omega = 56.4°$, $\phi = 37.2°$, $\kappa = 180°$. This peak was 6 σ, whereas the second-highest peak was less than 3 σ. Subsequent search for glutaminase domains corresponding to the three isomerase domains completed the protein model. The solution was inspected for inter-subunit contacts and subjected to rigid-body refinement (with each domain treated as a separate rigid body), which gave an R factor of 32% in the resolution range 15-4.0 Å.

The model was then refined with REFMAC.[27] Manual corrections of the model were introduced using the program O.[28] Electron density revealed the ten-residue linker between the two domains that was missing from the search model. At the end of refinement, water molecules were added in the $(F_o - F_c)$ map at the peaks of 3.5 σ, provided at least one hydrogen bond to the protein could be formed. The final R-free is 28%, R-overall is 20% for all data from 12 to 3.1 Å, and 80% of the resi-

Table 1. Data collection and refinement statistics

Data collection	
Resolution range (Å)	20.0-3.1
Wavelength (Å)	0.90
Measured reflections	111,045
Unique reflections	45,685
Completeness (%)	98.5
R_{merge} ($\Sigma\|I - \langle I\rangle\|/\Sigma I$)	0.056
$\langle I\rangle/\langle\sigma\rangle$ at d_{min}	2.8
B-factor from Wilson plot (Å^2)	74.5
Refinement	
R-factor	0.20
R-free (5% data)	0.28
Number of atoms	
Protein	14,085
Glc6P	32
Water molecules	39
r.m.s. deviation from ideality	
Bonds (Å)	0.015
Angles (deg.)	1.5
Mean B-factors (Å^2)	
Chain A	33.7
Chain B	66.3
Chain C	97.0

dues are in the most favoured region of the Ramachandran plot as defined in PROCHECK.[29] One residue, Glu91, is in a disallowed region. The same conformation of Glu91 ($\phi = 54.9$, $\psi = -106.5°$) was observed in the other structures of the glutaminase domain. Refinement statistics are given in Table 1. Crystallographic calculations were performed with the CCP4 suite.[30] Drawings were produced with O[23] and MOLSCRIPT.[31]

Protein Data Bank accession number

The structure has been deposited in the RCSB Protein Data Bank under accession number 1JXA.

References

1. Zalkin, H. & Smith, J. L. (1998). Enzymes utilizing glutamine as an amide donor. *Advan. Enzymol. Relat. Areas Mol. Biol.* **72**, 87-144.
2. Massiere, F. & Badet-Denisot, M.-A. (1998). The mechanism of glutamine-dependent amidotransferases. *Cell. Mol. Life Sci.* **54**, 205-222.
3. Raushel, F. M., Thoden, J. B. & Holden, H. M. (1999). The amidotransferase family of enzymes: molecular machines for the production and delivery of ammonia. *Biochemistry,* **38**, 7891-7899.
4. Borowski, E. (2000). Novel approaches in the rational design of antifungal agents of low toxicity. *Farmaco,* **55**, 206-208.
5. McClain, D. A. & Crook, E. D. (1996). Hexosamines and insulin resistance. *Diabetes,* **45**, 1003-1009.
6. Winslet, M. C., Poxon, V., Allan, A. & Keighley, M. R. (1994). Mucosal glucosamine synthetase activity in inflammatory bowel disease. *Dig. Dis. Sci.* **39**, 540-544.
7. Badet, B., Vermoote, P., Haumont, P.-Y., Lederer, F. & Le Goffic, F. (1987). Glucosamine synthetase from *Escherichia coli*: purification, properties, and glutamine-utilizing site location. *Biochemistry,* **26**, 1940-1948.
8. Badet-Denisot, M.-A., Rene, L. & Badet, B. (1993). Mechanistic investigations on glucosamine-6-phosphate synthase. *Bull. Soc. Chim. Fr.* **130**, 249-255.
9. Denisot, M.-A., Le Goffic, F. & Badet, B. (1991). Glucosamine 6-phosphate synthase from *Escherichia coli* yields two proteins upon limited proteolysis: identification of the glutamine amidohydrolase and 2R ketose/aldose isomerase-bearing domains based on their biochemical properties. *Arch. Biochem. Biophys.* **288**, 225-230.
10. Leriche, C., Badet-Denisot, M.-A. & Badet, B. (1996). Characterization of a phosphoglucose isomerase-like activity associated with the carboxy-terminal domain of *Escherichia coli* glucosamine-6-phosphate synthase. *J. Am. Chem. Soc.* **118**, 1797-1798.
11. Isupov, M. N., Obmolova, G., Butterworth, S., Badet-Denisot, M.-A., Badet, B., Polikarpov, I. *et al.* (1996). Substrate binding is required for assembly of the active conformation of the catalytic site in Ntn amidotransferases: evidence from the 1.8 Å crystal structure of the glutaminase domain of glucosamine 6-phosphate synthase. *Structure,* **4**, 801-810.
12. Teplyakov, A., Obmolova, G., Badet-Denisot, M.-A., Badet, B. & Polikarpov, I. (1998). Involvement of the C terminus in intramolecular nitrogen channeling in glucosamine 6-phosphate synthase: evidence from a 1.6 Å crystal structure of the isomerase domain. *Structure,* **6**, 1047-1055.
13. Teplyakov, A., Obmolova, G., Badet-Denisot, M. A. & Badet, B. (1999). The mechanism of sugar phosphate isomerization by glucosamine 6-phosphate synthase. *Protein Sci.* **8**, 596-602.
14. Thoden, J. B., Holden, H. M., Wesenberg, G., Raushel, F. M. & Rayment, I. (1997). Structure of carbamoyl phosphate synthetase: a journey of 96 Å from substrate to product. *Biochemistry,* **36**, 6305-6316.
15. Tesmer, J. J., Klem, T. J., Deras, M. L., Davisson, V. J. & Smith, J. L. (1996). The crystal structure of GMP synthetase reveals a novel catalytic triad and is a structural paradigm for two enzyme families. *Nature Struct. Biol.* **3**, 74-86.
16. Krahn, J. M., Kim, J. H., Burns, M. R., Parry, R. J., Zalkin, H. & Smith, J. L. (1997). Coupled formation of an amidotransferase interdomain ammonia channel and a phosphoribosyltransferase active site. *Biochemistry,* **36**, 11061-11068.
17. Knochel, T., Ivens, A., Hester, G., Gonzalez, A., Bauerle, R., Wilmanns, M., Kirschner, K. & Jansonius, J. N. (1999). The crystal structure of anthranilate synthase from *Sulfolobus solfataricus*: functional implications. *Proc. Natl Acad. Sci. USA,* **96**, 9479-9484.
18. Larsen, T. M., Boehlein, S. K., Schuster, S. M., Richards, N. G., Thoden, J. B., Holden, H. M. & Rayment, I. (1999). Three-dimensional structure of *Escherichia coli* asparagine synthetase B: a short journey from substrate to product. *Biochemistry,* **38**, 16146-16157.
19. Binda, C., Bossi, R. T., Wakatsuki, S., Arzt, S., Coda, A., Curti, B. *et al.* (2000). Cross-talk and ammonia channeling between active centers in the unexpected domain arrangement of glutamate synthase. *Structure,* **8**, 1299-1308.
20. Brannigan, J. A., Dodson, G., Duggleby, H. J., Moody, P. C., Smith, J. L., Tomchick, D. R. & Murzin, A. G. (1995). A protein catalytic framework with an N-terminal nucleophile is capable of self-activation. *Nature,* **378**, 416-419.
21. Badet, B., Vermoote, P. & Le Goffic, F. (1988). Glucosamine synthetase from *Escherichia coli*: kinetic mechanism and inhibition by N^3-fumaroyl-L-2,3-diaminopropionic derivatives. *Biochemistry,* **27**, 2282-2287.
22. Golinelli-Pimpaneau, B. & Badet, B. (1991). Possible involvement of Lys603 from *Escherichia coli* glucosamine-6-phosphate synthase in the binding of its substrate fructose 6-phosphate. *Eur. J. Biochem.* **201**, 175-182.
23. Rhee, S., Parris, K. D., Ahmed, S. A., Miles, E. W. & Davies, D. R. (1996). Exchange of K^+ or Cs^+ for Na^+ induces local and long-range changes in the three-dimensional structure of the tryptophan synthase $\alpha 2\beta 2$ complex. *Biochemistry,* **35**, 4211-4221.
24. Obmolova, G., Badet-Denisot, M.-A., Badet, B. & Teplyakov, A. (1994). Crystallization and preliminary X-ray analysis of the two domains of glucosamine 6-phosphate synthase from *Escherichia coli*. *J. Mol. Biol.* **242**, 703-705.
25. Otwinowski, Z. & Minor, W. (1997). Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* **276**, 307-326.
26. Vagin, A. & Teplyakov, A. (1997). MOLREP: an automated program for molecular replacement. *J. Appl. Crystallog.* **30**, 1022-1025.
27. Murshudov, G. N., Vagin, A. A. & Dodson, E. J. (1997). Refinement of macromolecular structures by

maximum-likelihood method. *Acta Crystallog. sect. D*, **53**, 240-255.
28. Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard, M. (1991). Improved methods for building models in electron density maps and the location of errors in these models. *Acta Crystallog. sect. A*, **47**, 110-119.
29. Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993). PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallog.* **26**, 283-291.
30. Collaborative Computational Project Number 4. (1994). The CCP4 suite: programs for protein crystallography. *Acta Crystallog. sect. D*, **50**, 760-763.
31. Kraulis, P. J. (1991). MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallog.* **24**, 946-950.

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